Proc. KSAM International Symposium
June 20-22, 2001, Chonan, Korea

Phosphorolytic Pathway in Cellulose Degradation

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Abstract

Two intracellular enzymes, cellobiose phosphorylase (CBP) and cellodextrin phosphorylase (CDP) are involved in the phosphorolytic pathway in cellulose degradation. Those enzymes are considered to be useful in syntheses of oligosaccharides because the reactions are reversible. CBP from *Cellvibrio gilvus* and CDP from *Clostridium thermocellum* YM-4 were cloned and over-expressed in *Escharichia coli*. Both the enzyme reactions showed ordered bi bi mechanism. Acceptor specificity of CBP in the reverse reaction was determined. Several ß-1,4-glucosyl disaccharides were synthesized by using the reaction. A new substrate inhibition pattern, competitive substrate inhibition, was also found in the reverse reaction of CBP. Cellobiose was produced from sucrose at a high yield by a combined action of three enzymes including CBP.

Introduction

Cellulose, the ß-1,4 linked glucose polymer, is definitely the most abundant biomass in the world. Considering the utility of the biomass, it is important to understand the degradation of cellulose occurring in the nature. Cellulose must be cleaved into glucose to enter the glycolytic pathway. Microorganisms that grow on cellulose have some enzyme systems to degrade cellulose.[1)]

Many microorganisms secrete a series of hydrolytic enzymes, cellulase, to cleave cellulose into fermentable sugars. Cellulase system represents the degradation of cellulose and has been well studied. Several kinds of cellulases having different substrate specificities are involved in the hydrolysis of cellulose by cooperative action.

It has been reported that some cellulase-secreting bacteria produce intracellular phosphorolytic enzymes for cellooligosaccharides. Two enzymes, different in their substrate specificities, are known as such phosphorolytic enzymes, cellobiose phosphorylase (CBP: EC 2.4.1.20) and cellodextrin phosphorylase (CDP: EC 2.4.1.49).

CBP has been found in several bacteria, especially in ruminal ones.[2)] CBP catalyzes the reversible phosphorolysis of cellobiose into glucose and a-glucose-1-phosphate. This enzyme is very specific to cellobiose. It does not hydrolyze cellooligosaccharides higher than DP 3 at all. Also it does not hydrolyze any ß-linked glucobioses other than cellobiose.

CDP has been found only in Clostridia, *Clostridium thermocellum* and *Clostridium sterocorarium*, accompanied with CBP[3)]. CDP catalyzes the reversible phosphorolysis of cellooligosaccharides greater than cellotriose at the non-reducing end to form a-glucose-1-phosphate. However, CDP does not phosphorolyze cellobiose.

Usually, microorganisms producing such enzymes secrete cellulase system that accumulates cellobiose as the final product. Accumulation of cellobiose may be advantageous for them because they can utilize cellobiose inside the cells using such phosphorolytic enzymes, but most of other microorganisms cannot utilize it.

CBP and CDP are often found in anaerobic bacteria. The reaction of CBP saves one molecule of ATP during glycolytic pathway that produces only 2 molecules of ATP. It is very important for anaerobic microorganism, but not so significant for aerobic bacteria because much more ATP can be produced through respiration.

These phosphorolytic enzymes are also useful in syntheses of oligosaccharides because the reaction is reversible. In this paper, we describe cloning, properties and utility of these enzymes.

Materials and Methods

Microorganisms: Cellvibrio gilvus (ATCC 13127) was used for the source of CBP. *Clostridium thermocellum* YM-4, a hyper cellullolytic strain, was the source of CDP. Both genes were cloned and expressed in *Escherichia coli* BL21 with pET28a vector.

Assay methods: One unit of the CBP was defined as the amount of the enzyme that produces 1 ole of G-1-P from 10 mM cellobiose and 10 mM phosphate at 37°C at pH 7.0. One unit of CDP was defined as the amount of the enzyme that produces 1 ole of phosphate from 10 mM G-1-P and 10 mM cellobiose. The amount of G-1-P was determined by the phosphoglucomutase / glucose-6-phosphate dehydrogenase method. That of Glc was done by glucose oxidase / peroxidase method. Inorganic phosphate was measured according to Lowry and Lopez[4] in the presence of G-1-P. Cellobiose in the reaction of CDP was quantified by cellobiose phosphorylase / glucose oxidase / peroxidase method.[5]

Results and Discussions

Cloning of CBP and CDP[6]

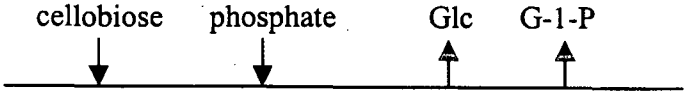
C. gilvus CBP gene was cloned based on the amino acid sequence of the purified CBP. The cbp gene encoded a protein with 822 amino acids and the calculated molecular mass of the deduced protein was 90,813, matching the molecular mass of the native protein determined to be 90,736+-248 by TOF-MASS. The expression of the recombinant CBP in *E. coli* BL21 with pET28a gave the active enzyme. Properties of the recombinant CBP were identical to those of the native CBP.

C. thermocellum YM-4 CDP gene was cloned based on the sequence similarity of CBPs and CDPs by using PCR techniques. The cdp gene encoded a protein with 984 amino acids and the molecular mass of the deduced protein was calculated to be 112,729. The expression of the recombinant CDP in *E. coli* BL21 with pET28a gave the active enzyme.

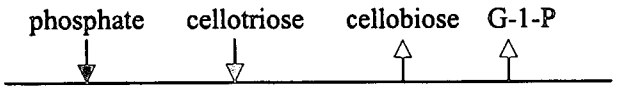
Until now, 5 cbp genes and 3 cdp genes have been cloned. A highly homologous region was found in the amino acid sequences of CBPs and CDPs.

Reaction mechanism of CBP and CDP[7]

The reaction mechanism of CBP was determined to be an ordered bi bi mechanism by investigating the inhibition pattern of both the products (G-1-P and Glc) toward both the substrates (cellobiose and phosphate). The result was as following: Cellobiose first bound to the enzyme and then phosphate bound to form a ternary complex. Phosphorolytic reaction took place in the ternary complex and the enzyme released Glc and G-1-P.

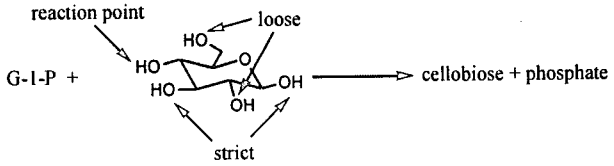


The reaction mechanism of CDP was also determined to be an ordered bi bi mechanism as shown below. In the case of CDP, phosphate bound to the enzyme before cellotriose bound. This result is different from that obtained with CBP. The order in the release of the products is the same with CBP.

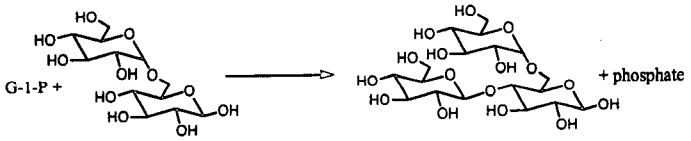


Substrate specificity of CBP[8)]

Substrate specificity of the acceptor molecule in the reverse reaction was studied. The enzyme recognizes glucose derivatives at position 2 and 6. It requires anomeric hydroxyl group of ß-configuration, even though the hydroxyl group is so apart from the reaction point.



Several ß-1,4-glucosyl disaccharides were synthesized by using CBP. Branched trisaccharides were also synthesized by using 1,6-linked disaccharides such as gentiobiose, isomaltose and melibiose as the acceptor molecule.

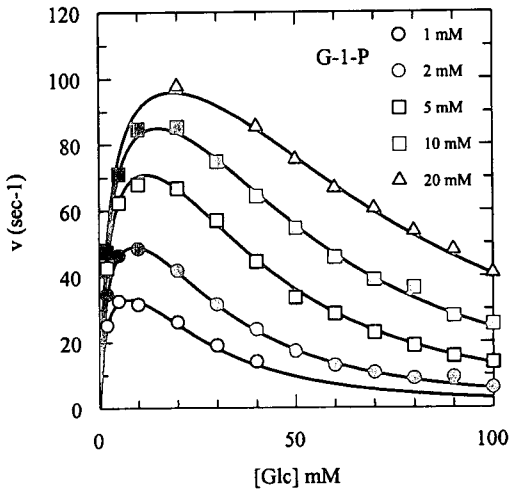


isomaltose (Glc1-6Glc) Glc1-4(Glc1-6)Glc

Competitive substrate inhibition in the reverse rdaction by CBP[9)]

It is found that an increase in Glc concentration caused a decrease in tje reaction rate above a certain concentration. This phenomenon is explained by the following concept:

Glucose, a substrate of CBP, competes with G-1-P, the other substrate, at the G-1-P site of CBP.



Practical production of cellobiose by using CBP[10]

Sucrose was converted into cellobiose by the action of three enzymes, sucrose phosphorylase, xylose isomerase, and CBP in the presence of a catalytic amount of phosphate as described below.

Sucrose + phosphate	⟷	G-1-P + Fructose	(sucrose phosphorylase)
Fructose	⟷	Glc	(xylose isomerase)
G-1-P + Glc	⟷	Cellobiose + phosphate	(cellobiose phosphorylase)
Sucrose	⟷	Cellobiose (Three enzymes + catalytic amount of phosphate)	

The reaction yield was more than 88% from sucrose. Starting from 500 g/l sucrose, cellobiose formed was automatically crystallized in the reaction mixture. This phenomenon is useful to use this method as semi-continuous process. Finally, crysralline cellobiose whose purity was greater than 98% was produced from sucrose at 90% yield repeating the reaction 7 times.

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